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PRELIMINARY COMMUNICATIONS

CYTOSOL-MEDIATED REDUCTION OF RESORUFIN FLUORESCENCE: EFFECTS ON THE ETHOXYRESORUFIN O-DEETHYLASE (ETR) ASSAY

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The assay for deethylation of ethoxyresorufin (ETR) by hepatic subcellular fractions has achieved significant use because it is easy to perform and seems to be quite specific for the cytochrome(s) P-448 (P_1-450) (1). It has been employed to characterize the inducing abilities of various compounds (2) as well as to determine whether individual animals are inducible at the Ah locus (3). Although the assay has typically been performed with microsomes (1,2,3), it has also been employed with hepatic homogenates from rats and chickens (4,5). While attempting to employ the ETR assay to characterize a rat 9,000 x *g* supernatant, to be used as an exogenous source of metabolic activation in bacterial mutagenesis and mammalian transformation assays, we observed enzymic activity in the cytosol which interfered with the assay.

MATERIALS AND METHODS

Chemicals. Resorufin and ethoxyresorufin were generously provided by Dr. R. Mayer (Texas A&M, College Station, TX). NADPH was purchased from Boehringer/Mannheim, Indianapolis, IN. Aroclor 1254 was obtained from Analab, North Haven, CT.

Preparation of samples. Male Fischer rats were induced by a single intraperitoneal injection of either corn oil (0.5 ml) or Aroclor 1254 (500 mg/kg body weight) five days prior to being killed by CO_2 asphyxiation. Livers were removed and were homogenized in 0.25 M sucrose-0.05 M Tris, pH 7.5. This and all remaining steps were performed at 4°C. Homogenates were clarified by spinning at 9,000 x *g* and the resulting supernatant (designated S-9) was either assayed directly or was recentrifuged at 105,000 x *g*, yielding a supernatant (designated cytosol) and a pellet which was resuspended in a volume (equivalent to that of the cytosol) of 0.25 M sucrose-0.05 M Tris, pH 7.5, and designated microsomes.

ETR assay. Resorufin formation was measured at room temperature by placing 25 μl of S-9, microsomes or cytosol (0.79, 0.18 and 0.62 mg of protein, respectively) in a quartz cuvette containing 2.0 ml of 0.05 M Tris, pH 7.5, and 1.7 x 10^{-6} M ethoxyresorufin. The reaction was started by adding 10 μl of 100 mM NADPH to the cuvette. Resorufin fluorescence was determined by exciting at 522 nm and measuring fluorescence at 586 nm.

Reduction of resorufin fluorescence. Reduction of resorufin fluorescence was measured at room temperature by placing 20 μl (0.49 mg protein) of hepatic cytosol into 2.0 ml of 0.05 M Tris, pH 7.5, containing 1.56 μM resorufin. The reaction was measured either in the presence or absence of 500 μM NADPH.

RESULTS AND DISCUSSION

Initial measurement of ETR activity in the Aroclor-induced 9,000 x *g* supernatant fraction (S-9) showed kinetics which rapidly deviated from linearity (Figure 1A). These non-linear kinetics were observed following alterations in the assay, i.e. (1) a 3-fold increase in NADPH, or (2) a 3-fold increase in ethoxyresorufin, or (3) a decrease in S-9 protein (R. W. Nims and R. A. Lubet, unpublished). We then measured ETR activity in a microsomal suspension containing the same amount of microsomal protein (albeit less total protein) from the same Aroclor-induced rat hepatic preparation. These results showed a much more

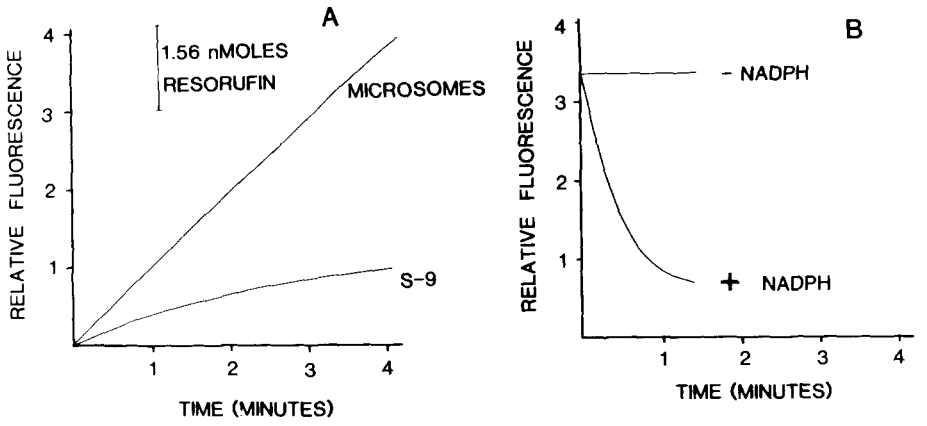

Fig. 1. (A) Resorufin fluorescence following deethylation of ethoxyresorufin. (B) Reduction of resorufin fluorescence by rat hepatic cytosol.

rapid and linear rate of formation of resorufin (Figure 1A). No ETR activity was detected in the Aroclor-induced cytosol alone, or in the corn oil-induced microsomal, S-9 or cytosolic preparations (data not shown). These results implied that there was something in the Aroclor induced cytosol which interfered with the ETR assay. We therefore measured the effect of the cytosols on fluorescence of the product resorufin (Figure 1B). We found that the Aroclor-induced cytosol reduced resorufin fluorescence in the presence of NADPH but not in its absence. The corn-oil induced cytosol did not appear to reduce resorufin fluorescence in the presence of NADPH (data not shown). We have observed similar non-linear kinetics when performing the ETR assay with Aroclor-induced hepatic S-9 from the hamster (R. W. Nims and R. A. Lubet, unpublished). We have subsequently observed that the reduction of resorufin fluorescence is dicumerol sensitive, bears many of the characteristics of cytosolic quinone reductase (EC 1.6.99.2) and may represent a sensitive assay for this enzyme (R. W. Nims, R. A. Prough, R. E. Kouri and R. A. Lubet, manuscript in preparation). In summation, we have observed that the standard ETR assay cannot be performed with an Aroclor-induced 9,000 x *g* supernatant fraction (S-9) and that this inability is due to the presence of a cytosolic component which reduces the fluorescence of the product in the presence of NADPH. This same cytosolic activity is likely to similarly affect the assay when used to determine ETR activity in Aroclor-induced intact rat hepatocytes or hepatocyte homogenates.

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